OOCYTE DEVELOPMENT AND INCORPORATION OF H^3–THYMIDINE AND H^3–URIDINE IN PECTINARIA (CISTENIDES) GOULDII

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Transformation from the oogonium and development of the primary oocyte involves intricate morphological and metabolic changes in the cell. Among these are enlargement of the germinal vesicle, development of the nucleolus and cytoplasmic growth (Raven, 1961). It is generally believed that the necessary premeiotic changes in the nucleus precede these phenomena.

Most evidence indicates that H^3-thymidine is not incorporated into the germinal vesicle of the mature oocyte (Ficq, 1961a; Favard-Séréno and Durand, 1963b; Ficq, Aiello and Scarano, 1963) although recently Holland and Giese (1965) report that both the oogonia and the pre-leptotene primary oocytes of the sea urchin synthesize DNA within the ovary. These results imply that the synthesis of DNA needed for subsequent growth and maturation of the oocytes must occur after the last oogonial division and no later than the early premeiotic changes in the oocyte nucleus.

Since there is evidence that various types of eggs have vast reserves of cytoplasmic DNA (Bieber *et al.*, 1959) or deoxyribosides (Hoff-Jørgensen and Zeuthen, 1952) and some indication of cytoplasmic uptake (Ficq, 1961a; Gintsburg, 1963) of H^3-thymidine into the oocytes, the question arises, is the entire cytoplasmic reserve acquired at the time of nuclear DNA synthesis or is it augmented throughout development of the oocyte? It is pertinent to determine whether the developing and growing oocyte is metabolically stable or if it is able to actively increase its DNA reserve. The continuous presentation of a specifically labeled nucleoside, H^3-thymidine, *in vivo* is one approach to this problem.

In contrast, the uptake of RNA precursors into the maturing oocyte appears to be widespread during oogenesis. In the starfish, H^3-uridine uptake is localized in the nucleus, nucleolus (Ficq, 1961b) and cytoplasm (Geuskens, 1963), in the nucleus and nucleolus of the sea urchin oocyte (Ficq, Aiello and Scarano, 1963) and the nuclear sap, chromosomes and cytoplasm of the cricket oocyte (Favard-Séréno and Durand, 1963a). The use of H^3-uridine and other precursors in conjunction with various inhibitors implicates the synthesis of at least three types of RNA (m-RNA, r-RNA and t-RNA) during maturation of the starfish and sea urchin oocyte (Ficq, 1961a; 1962; 1964).

Since both the nucleolus and nucleus in *Pectinaria* increase in size and activity as the oocyte develops (Tweedell, 1962), a study of the incorporation of labeled uridine into the RNA of the nucleolus and nucleus seemed promising (Tweedell, 1964).

Fortunately, the growth and development of the primary oocyte in *Pectinaria* occurs in the coelom after the cells leave the ovary. The utilization of these labeled precursors can be observed *in vivo* within the ovary and during individual stages of oocyte formation in the coelom. These results are compared with *in vitro* exposure of the oocytes to the same nucleosides.

Materials and Methods

Specimens of the marine polychaete annelid *Pectinaria* (= *Cistenides*) *gouldii* Verrill (Hartman, 1941) were obtained from the north shore of Cape Cod during June, July and August of 1963 and 1964. They were maintained in the laboratory as described earlier (Tweedell, 1962).

Incorporation of isotopes

In vivo. Before labeling, the animals were carefully removed from their sand test and the sex was determined microscopically by identification of the gametes beneath the ventral body wall or the parapodia. The animals were then narcotized with 50% ethyl alcohol added dropwise to a small dish of sea water until the animals were flaccid. Isotopes were injected with a #27 hypodermic needle directly through the cephalic plaque (Fig. 1) of the animals into the coelomic cavity. Narcotized animals were injected according to size with either 5 to 10 μC. of tritiated thymidine (1.9 C./millimole) or 5 to 10 μC. of tritiated uridine (1.7 C./millimole). The addition of a small amount of Nile blue sulphate indicated that most of the injected fluid was retained. The animals were then returned to their tests and placed in normal sea water where they recovered quickly. Animals were sacrificed or the eggs were harvested and fixed at intervals of $\frac{1}{2}$, 1, 2, 4, 8, 16, 24, 48 and 72 hours. Additional egg harvests were made at 2, 3, 6–7 and 19–21 days after injection. At least two animals were examined for each of the above intervals.

In vitro. Gametes were also shed directly into sea water containing 10 μC. of H^3-thymidine or H^3-uridine per ml. of sea water. Following germinal vesicle (G.V.) breakdown of the mature oocytes, they were removed, washed and fixed at intervals of 2, 4 and 8 hours after exposure. Thereafter they were embedded in paraffin and sectioned at 4 μ.

Recovery of free oocytes. Some of the animals injected *in vivo* were shed and the eggs immediately fixed as whole mounts on coverslips with Kahle's fixative using a modified double coverslip sandwich technique (Tweedell, 1962). Similar treatment was given to oocytes which were labeled by shedding directly into sea water containing the isotopes. At the moment of fixation, the cells were compressed by placing 45 grams pressure on the coverslips to further flatten the oocytes. After fixation the whole mounts were washed and dehydrated to 100% ethyl alcohol when they were air dried. The coverslips were then cemented to slides with the eggs facing upward. Equivalent animals were fixed at the same time for future exposure of the oocytes in sections.

Preparation of intact animals. The majority of the oocytes labeled *in vivo* were processed for sectioning *in situ*. Prior to histological preparation of adult animals it was necessary to remove the sand grains from the digestive tract. Origi-

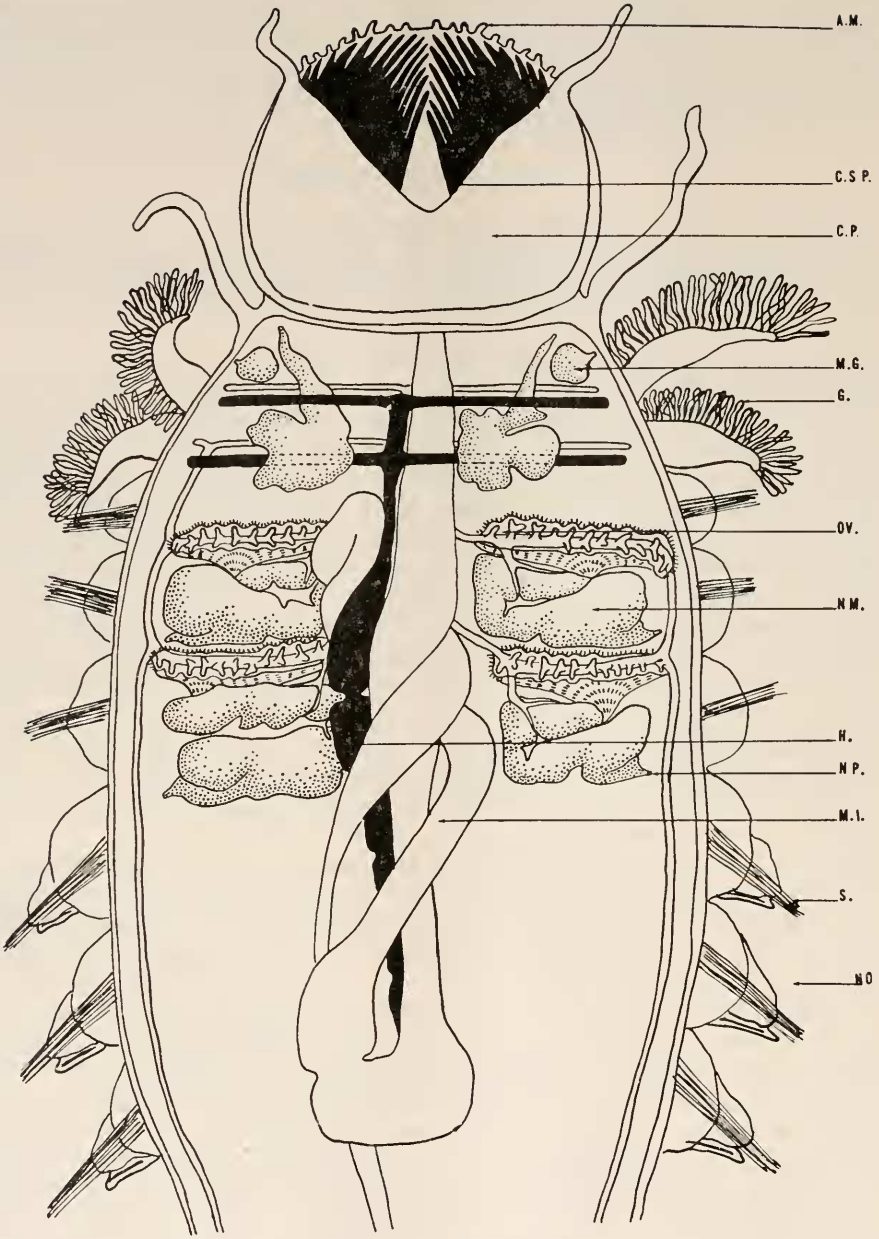


FIGURE 1. A dorsal dissection, cut in the midline and spread laterally, of an adult *Pectinaria* (♀), showing position of the ovaries and attached nephromixia. CSP, cephalic spines; CP, cephalic plaque, AM, antennular membrane; G, gill; NM, nephromixium; NP, nephrodiopore; MG, mucus gland; OV, ovary; NO, notopodium; S, setae; MI, middle intestine; H, heart.

nally, the technique of Hanks (1960) was repeated, in which he fed living animals ground quahog shells. As reported, the animals readily ingested the calcium carbonate. However, the required decalcification by acetic acid in the *intact* animal was not successful and was further complicated by the collection of CO_2 which could not be removed under vacuum. Alternate materials, dried coffee grounds, corn meal, powdered charcoal, ground rice, Dowex resin and various household cereals were tested. In all cases, the materials were ground and screened through wire mesh to the equivalent size of large sand grains. These materials were placed in 5-inch open glass tubes, $\frac{1}{2}$-inch diameter, that were covered at one end with several layers of cheesecloth. The tubes were half-filled with the test material and placed cloth end down in a test tube rack. The adult animal was inserted head down in the tube and a siphon tube of slowly running sea water was introduced at the top to insure a constant sea water flow. Animals were allowed to "feed" in this position for 24 to 48 hours, then removed and left to "work" in running sea water for 24 hours while excess ingested products were eliminated. Three materials were successful: powdered charcoal, ground white rice grains and a protein cereal (Kellogg's Concentrate). The latter two were readily ingested and perfectly compatible with histological sectioning while the charcoal sections tended to crumble. After removal from their tests the adult animals were narcotized and then fixed in Kahle's or Kleinenberg's fixative, double embedded in methyl benzoate-parlodion paraffin and sectioned at 5 μ.

Autoradiography. Both compressed whole oocytes and sections were prepared for autoradiography by dipping the slides in Kodak NTB2 or Ilford G-5 emulsions (diluted 50/50 with distilled water). The slides were incubated in light-tight boxes with "Drierite" at 4° C. for the duration of exposure from two to three weeks. Slides were developed in D-72 or D-19 for 3 minutes at 14° C., placed in stop bath for 15 seconds and fixed for 3 minutes at 14° C. The slides were then washed for $\frac{1}{2}$ hour in running water.

Coverslip mounts were stained in gallocyanin chrome-alum, pH 1.7, for 8–12 hours and counterstained with eosin. Sections were stained in Galligher's haematoxylin, eosin-azure II (Gurr) or Jenner-Giemsa solution.

Serial sections of oocytes retained within the whole animal or sections of shed eggs were treated with RNase or DNase prior to autoradiography. DNase was applied to sections alternating with control sections in a concentration of 0.1 mg./ml. prepared in 0.05 *M* Michaelis veronal acetate buffer, pH 6.8 with 0.0025 *M* $MgCl_2$ added. Incubation was at 37° C. for 2 hours.

RNase was prepared as 3 mg./ml. in 0.05 *M* Sorensen phosphate buffer at pH 7.6. The sections were incubated in RNase for 2 hours at 48° C., along with non-enzyme-treated control sections.

Observations

The ovaries are minute, well vascularized organs located along the anterior surface of two pairs of prominent yellow brown organs, identified as nephromixia (Goodrich, 1945). Externally, the paired nephromixia can be seen through the lateral body walls of the 2nd and 3rd setae-bearing segments. The latter glands, attached to the ventral body wall (Fig. 1) first loop dorsally at the midline, then

open externally through dorso-lateral nephridiopores which are slightly caudal to the abbreviated setae of the parapodia on the same segments.

Viewed from a dorsal dissection, the ovaries are seen anterior and slightly dorsal to the nephromixia. The ciliated ovaries are narrow transparent organs enveloping lateral ovarian blood vessels which flow along the cephalic edges of the nephromixia. Lobe-shaped fins projecting at right angles to the main axis of the ovary extend dorsad and somewhat anteriorly from the ovaries. These fins are sinuses confluent with the ovarian vessel and are also covered with cilia.

The germinal portion of the ovary consists of budding areas located at the ventral medial end of each of four ovaries (Figs. 2, 3). Sometimes an additional area is found at the dorsal lateral end. The ovarian cytology seems to vary according to the season. Early in the summer the ovaries are largely composed of interphase oogonial cells and mitotic figures are common, whereas in August the ovaries have a large proportion of oocytes in the leptotene stage (Fig. 4).

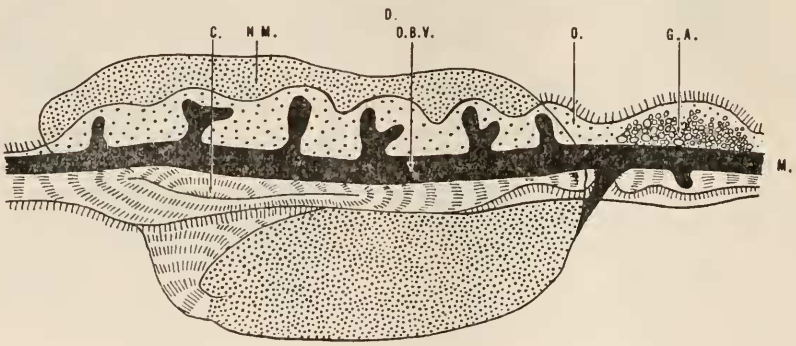


FIGURE 2. An anterior view of a single nephromixium and attached ovary (as seen in Figure 1). D, dorsal; M, medial; C, coelomostome; NM, nephromixium; OV, ovary, enclosing lateral ovarian vessel; GA, germinal area.

The generative phase (Raven, 1961) of oocyte formation begins within the germinal area of the ovary. The oocytes are budded off directly into the coelomic cavity from the germinal area at the cephalic free edge of the ovaries. Thus a sample of the coelomic fluid from an adult female yields a series of developmental phases in the formation of the primary oocytes after they leave the ovaries. The developing oocytes are moved and bathed continuously in this fluid by the muscular pumping of the adult. A diverse selection of cell types: amoebocytes, gregarines, histiocytes and others, are included in the coelomic fluid.

Two development phases of oocyte formation within the coelom can be recognized, a packet phase that is followed by an individual oocyte (solitary) phase. The packets consist of small cell clumps, about 18 to 20 microns in diameter, arranged about a central core area. They appear in the coelomic fluid after being budded from the surface of the ovary (see Fig. 3).

There usually are 16 to 32 oocytes within a packet and the individual cell is about 5.5 microns in diameter. The nucleus fills the cell, leaving a thin rim of cytoplasm, and the chromosomes are generally in the zygotene stage. The single

nucleolus is about twice the size of those within the ovary. Thereafter the packets slowly increase in size from growth of the individual cells and a small "core" forms in the packet center. The enlarged nuclei, entering the pachytene stage, now have a dominant nucleolus and the cytoplasm shows heavy basophilia (stainable with alcian blue, gallocyanin and toluidine blue) at the basal ends of the cells near the center of the packet. The largest packets consist of loosely bound cells each measuring 10 to 20 microns in width.

During the second growth or vegetative phase, the packets fragment and individual oocytes appear in the coelomic fluid, the smallest measuring from 10 to 12.5 microns. These cells are distinguished by a large germinal vesicle and a single amphinucleolus. The increase in size of these solitary oocytes is due to both nuclear and cytoplasmic growth. The mature oocyte is a flattened ellipse which averages 48 by 56 microns and, since it is flattened, about 25 microns in thickness. At this time the germinal vesicle has an average diameter of 40 microns. Thus, using the cell diameter as a crude measurement of linear growth, the cells are seen to increase about ten times in size since budding from the ovary.

As the oocyte approaches full development, the diplotene chromosomes are conspicuously distributed throughout the germinal vesicle. Increased activity of the nucleolus is evidenced by the formation of epinucleolar and intranucleolar buds.

Mechanical stimulation of the adult usually causes artificial shedding when part or all of the coelomic fluid with its cellular components is ejected from posterior coelomoducts. Approximately 12 to 18 minutes after shedding into sea water (Austin, 1963; Tweedell, 1962) the fully developed oocytes undergo germinal vesicle breakdown, become spherical and develop to the metaphase of the first maturation division where they remain unless fertilized.

There are indications, however, that the natural spawning process follows a different sequence of events. During late summer induced shedding occasionally produces oocytes that are already in the first maturation division. However, these cells are ejected from the nephridiopores. Ordinarily, the nephromixia are relatively empty, but during late summer their distal ends contain heavy concentrations of oocytes. Coelomic oocytes are drawn into the nephromixium along a heavily ciliated groove that lies on its anterior edge. The ciliary action transports the oocytes laterally to a large ciliated funnel (coelomostome) through which they enter the nephromixium (Fig. 2). Upon stimulation, these oocytes are readily observed leaving the nephridiopores.

In addition, sections through the intact animal reveal gravid nephromixia in which the enclosed oocytes are almost exclusively in a post-G.V. state while the germinal vesicles of fully developed oocytes within the coelom are still intact (Fig. 5). Presumably, during the natural spawning process G.V. breakdown occurs within the nephromixia and the oocytes are shed through the nephridiopores.

Some of the cells within the nephromixium after germinal vesicle breakdown are often still in late diakinesis (Fig. 6). Chromosome bivalents are widely spread throughout the cytoplasm and the nucleolus is absent. Generally it is possible to distinguish 16 large bivalents, yet as many as 9 additional obscure, weakly staining doublets can be seen. Austin (1963) reports from 17 to 21 bivalents from his observations, the variation being attributed to faint, minute chromosomes. Obviously a detailed karyotype study is needed. In other cells within the nephro-

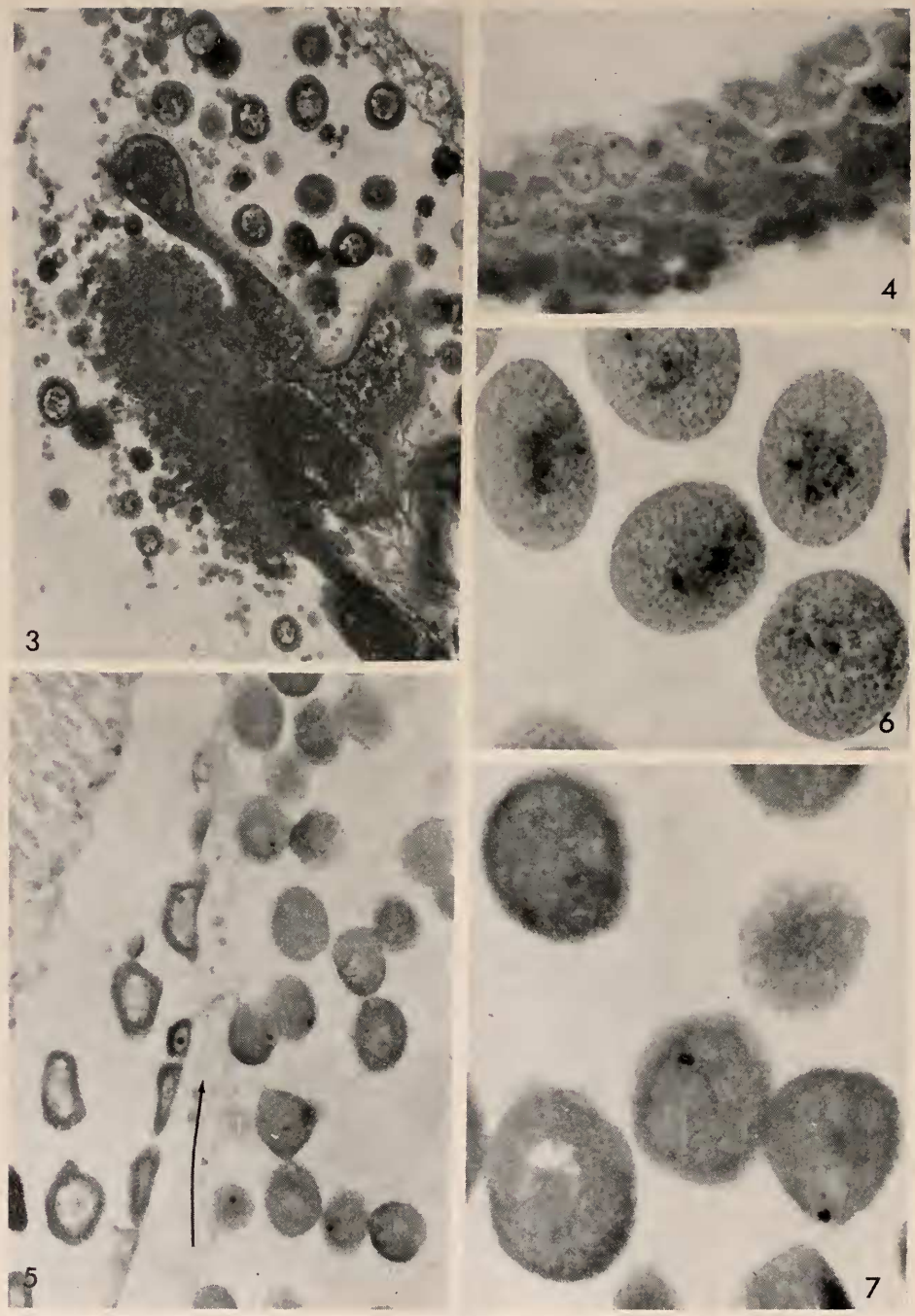


PLATE I

mixium, the chromosomes are condensed into a small knot, where they remain arrested in the first maturation division.

Results

H^3-thymidine

In vivo labeling. Following injection of tritiated thymidine into the living adult, oocytes were harvested by shedding and prepared as whole mounts or embedded and sectioned. Entire animals were also embedded and sectioned intact.

The ovary. Tritiated thymidine was quickly incorporated into the ovary after injection into the coelom. Localization was principally over the nuclei of cells scattered throughout the ovary. The first uptake was detected two hours after contact with H^3-thymidine (Fig. 8). The labeled cells were generally found deep in the mid region of the ovary rather than at the free surface. Since the label in the ovary often appeared above the nuclei of cell pairs, these cells presumably had just completed cell division and the resultant oocytes were exposed to thymidine during the period of DNA synthesis (S period). Oocytes associated with the budding surface of the ovary did not show appreciable nuclear label at this time.

After 12 hours exposure to the thymidine some of the deep-lying cells of the ovary were intensely labeled while those along the surface were more lightly marked (Fig. 9). Scattered labeled cells appeared throughout the ovary and extended to the free border where they budded off. Labeled oocytes were also observed in ovaries recovered from animals 24 and 48 hours after injection of H^3-thymidine with no essential difference.

Heavy nuclear labeling was still evident in ovarian oocytes taken from animals after one-week exposures. The major change from earlier ovarian sections was the presence of more labeled cells at the free surface of the ovary (Fig. 10). Recently released labeled oocytes were often seen adjacent to the ovary. In general the labeled oocytes were more widely distributed but less heavily tagged over the nuclei.

After 21 days exposure to a single pulse, grains were diffusely scattered over the remaining intact oocytes. This distribution suggested that the incorporated thymidine had been diluted out by subsequent mitoses of the remaining oogonia.

The actual length of the synthesis period prior to oocyte growth and development was undetermined but H^3-thymidine incorporation in the ovary did indicate when DNA synthesis took place. The assumption was then made that the cells which incorporate thymidine after a short pulse time were either oogonia or pre-

PLATE I

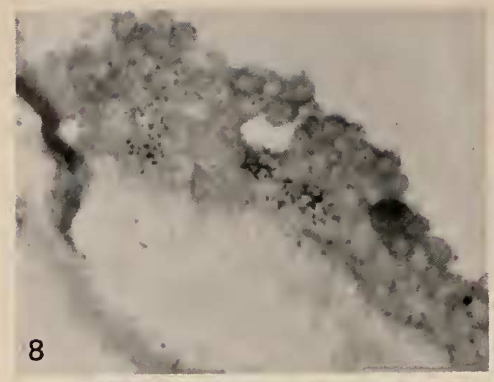
Figure 3. Cross-section of a normal ovary and attached ciliated lobe. Oocytes are budding from free edge of ovary at lower left.

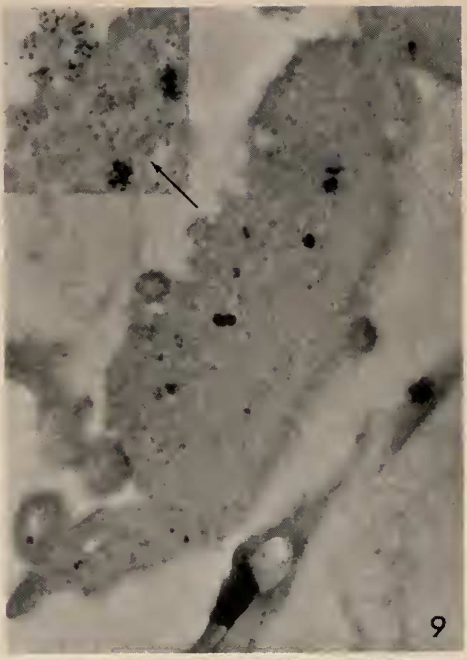
Figure 4. Developing primary oocytes in premeiotic stages within the ovary. 1920 ×.

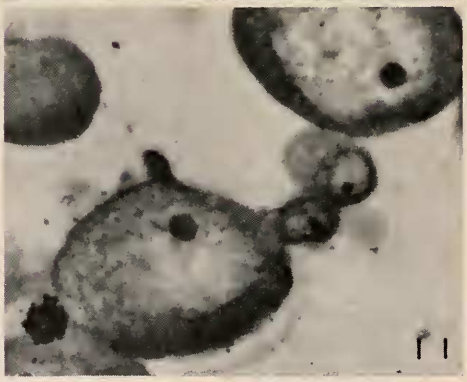
Figure 5. Post-G.V. oocytes within lumen of nephromixium on the right; pre-G.V. coelomic oocytes to the left. Arrow indicates wall of nephromixium separating pre- and post-G.V. oocytes. H^3-thymidine-injected, 2-hour exposure. No uptake in either pre-or post-G.V. oocytes. 350 ×.

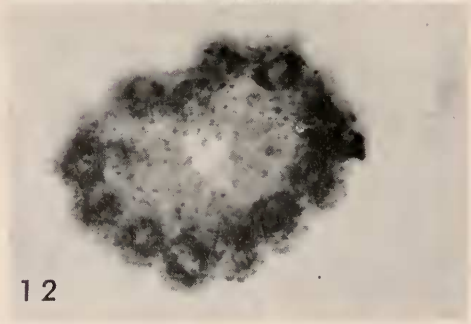
Figure 6. Post-G.V. oocytes from within nephromixium of control animal. Optical plane shows portion of chromosomes in diakinesis. 1200 ×.

Figure 7. Post-G.V. oocytes in nephromixium showing absence of H^3-thymidine incorporation after a two-hour pulse. 580 ×.









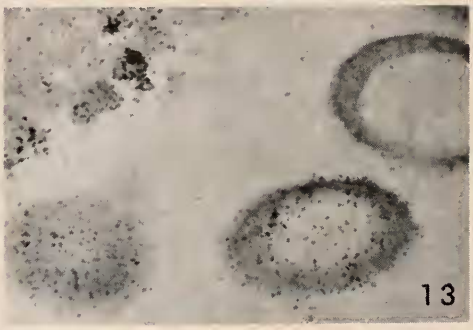


PLATE II

meiotic oocytes in the "S" period preceding growth and development of the oocyte. Consequently, the developmental age of subsequent stages found with nuclear label was extrapolated at successive intervals after injection; the next most advanced stage so labeled indicated its developmental age.

Coelomic oocytes. After a short exposure period, thymidine was also readily incorporated into the oocyte packets of the coelom. The especial difference between the ovarian oocytes and the oocyte packets was in the localization of the radioactivity. The packet oocytes were mainly labeled above the cytoplasm with relatively little nuclear label.

The smallest coelomic oocyte packets, containing cells of a size comparable to ovarian oocytes, began showing a label primarily over the cytoplasm after a pulse of 30 minutes. Thereafter, recovery of shed oocytes from animals at intervals of 2, 4, 8, 16 or 24 hours after injection revealed the same pattern of labeling. An example of a packet recovered after 24 hours from an animal injected with 5 μC. is seen in Figure 11. The nuclei of these small packet cells were in the zygotene stage. Larger cell packets and single oocytes were noticeably unlabeled. Sections of oocytes *in situ* from a parallel series of injected animals demonstrated identical uptake in the very early packets of oocytes recovered up to 24 hours after injection.

As it was not possible to effectively chase the tritiated thymidine from the coelom of the living animal, it was not certain whether these early cell packets incorporated the thymidine before or after leaving the ovary. The latter possibility was most likely since ovarian oocytes at the budding edge of the ovary did not become labeled until after 12 hours injection. Thus, these small packets were distinctive since they were the only coelomic oocyte stages found with a significant grain count after 2 to 24 hours exposure to tritiated thymidine.

Examination of coelomic oocytes sectioned *in situ* after an exposure of two days indicated a significant change in the labeling of cell packets. A few larger oocyte packets, presumably resulting from cellular growth of younger packets, began exhibiting a heavy cytoplasmic label with very few grains over the nucleus. The additional labeling of older oocyte packets suggested that the developing oocytes had acquired the H^3-thymidine during the early packet stage and that cytoplasmic and nuclear growth of the individual oocytes followed subsequently.

Both small and medium-size oocyte packets continued to show a predominately cytoplasmic label up to 72 hours after exposure to labeled thymidine (Fig. 12); others had a light nuclear label.

PLATE II

FIGURE 8. Edge of ovary after a two-hour exposure to H^3-thymidine. Label occurs over isolated cells along the interior.

FIGURE 9. Ovary from H^3-thymidine-injected animal sacrificed after 12 hours. Scattered oocytes are heavily labeled over the nuclei. The free budding edge of ovary is at the left. A small labeled oocyte packet is seen in insert. 234 ×.

FIGURE 10. A section of ovary from a H^3-thymidine-injected animal sacrificed after 7 days, showing scattered distribution of labeled oocytes. 585 ×.

FIGURE 11. Single coelomic oocytes which are unlabeled after a two-hour pulse of H^3-thymidine. A heavily labeled oocyte packet is seen at the lower left. 375 ×.

FIGURE 12. A medium-size packet of developing oocytes from an animal sacrificed 72 hours after H^3-thymidine injection. Incorporation is principally over the cytoplasm.

FIGURE 13. Large single developing oocytes recovered 7 days after H^3-thymidine injection. A heavy label finally appears over the nucleus and cytoplasm. The edge of the ovary appears on the left. Note that *not* all of the large oocytes are labeled. 585 ×.

After a prolonged exposure of 7 days, many small and medium oocyte packets were labeled as they had been earlier. More significantly, new oocyte packets with a heavy nuclear label appeared. Since cell division does not occur until germinal vesicle breakdown, these packets must have been labeled and subsequently released from the ovary.

Animals which had been injected with H^3-thymidine were also sacrificed after 21 days. Both small and medium cell packets and a few late oocyte packets were found with evidence of thymidine uptake. In the small packets the grains were scattered over the cytoplasm but the heaviest concentration occurred over the nucleus. These packets possibly represented cells that had taken thymidine into the nucleus at an earlier time and the labeled material had moved into the cytoplasm secondarily. Alternate oocyte sections from one animal exposed *in situ* for 21 days were pretreated with DNase prior to emulsion dipping. The DNase removed both the nuclear and cytoplasmic label from the oocyte packets.

Single oocytes. Individual coelomic oocytes in the final growth phase failed to indicate any incorporation of H^3-thymidine whether they were harvested early ($\frac{1}{2}$ to 16 hours), as seen in Figure 11, or after prolonged exposure (1 to 2 days). The same results were obtained from compressed egg whole mounts and from a study of sections of 8 entire animals sacrificed at 2, 12 or 24 hours after injection with either 5 or 10 μC. of tritiated thymidine. The solitary, small immature coelomic oocytes were completely unlabeled in all material harvested up to a day after injection. The larger mature cells also failed to take in the precursor. It was also found that mature oocytes, in metaphase I, within the nephromixium showed a complete absence of any labeling (Fig. 7).

Occasional single oocytes, in their early growth phase, were not significantly tagged until 48 to 72 hours after injection. In these cases the grains were evenly distributed over the entire cell. The majority of these single oocytes, however, remained unlabeled. A sampling of 50 fields from different slides showed an average of 9% of the larger, single oocytes were tagged at 72 hours. The average number of grains per labeled cell was 196 as compared to a background count of 7 from an equivalent area.

Since even the smallest single oocyte failed to show uptake of thymidine after a pulse of 30 minutes, it was assumed that the small number of older oocytes were labeled at the packet stage or earlier. Therefore, the minimal time for an oocyte to reach full size appeared to be from 48 to 72 hours.

Isolated single oocytes, recovered at still longer periods after injection gave further evidence of thymidine redistribution. A study of three different H^3-thymidine-injected animals sacrificed and sectioned 7 days after injection showed again that individual maturing oocytes were labeled. The marked cells were few and scattered, and the grains appeared with equal intensity over the entire cell. In many cases a heavily labeled oocyte occurred adjacent to a group of entirely unlabeled mature oocytes (Fig. 13). Sections through the center of the oocyte indicated little if any of the label was concentrated in the nucleus. This also suggested that the radioactive material had entered the cell during an earlier packet phase.

In animals sacrificed after 21 days individual oocytes of increasing sizes were also tagged. Many of the larger oocytes were still unlabeled but a survey of 50

fields indicated that an average of 17% of the largest oocytes had the labeled precursor. The grain count averaged 228 per cell compared to 9 for an equal area of background. It was apparent that some of these oocytes approaching maturity were more lightly labeled than others. The grains were distributed evenly over the nucleus and the cytoplasm in the more heavily labeled cells. Some of the lighter marked cells showed grains only over the cytoplasm.

In vitro labeling. The previous results demonstrated the inability of solitary developing coelomic oocytes or mature oocytes to incorporate H^3-thymidine directly into the nucleus but indicated a possible cytoplasmic uptake of thymidine. Conceivably, the events associated with the breakdown of the germinal vesicle and entrance of the cell into metaphase I could have altered the pattern of thymidine incorporation; this seemed to justify further tests on the oocytes after G.V. breakdown.

Direct shedding of the oocyte stages into H^3-thymidine provided a more concise measure of the time of uptake into the early packet stages. The absence of the ovary assured that the earliest stages were post-ovulatory and relatively short pulse times could be tested after adequate washing, assuring that appearance of a label over a particular stage would represent actual uptake at that developmental stage.

Oocytes were shed directly into sea water containing the precursors, and by regulating the time of initial exposure to the labeled compounds, it was possible to compare the mature primary oocytes before, during and after G.V. breakdown. Initial exposure to the labeled compounds was made from 5 minutes after shedding (prior to G.V. breakdown) and up to one hour post-shedding (after G.V. breakdown). The cells were usually pulsed for two hours but in some cases were left in the isotopes for 10 to 12 hours.

Oocytes placed into 5 or 10 μC. of thymidine/ml. of sea water between 5 and 10 minutes after shedding always possessed intact germinal vesicles at the time they were introduced. After one or two hours they were washed, fixed and prepared as whole mounts or embedded and sectioned.

In some cases G.V. breakdown did not occur in any of the full grown oocytes but this was attributed to a lack of maturity. In other experiments up to 80% of the cells showed G.V. breakdown. In neither instance did any of the single oocytes show any nucleoside incorporation. Prolonged exposure of the oocytes for 10 to 12 hours had no effect.

Oocytes were also shed into plain sea water, where mature oocytes underwent G.V. breakdown. After 20 minutes they were placed into sea water containing H^3-thymidine. The results were the same. None of the post-G.V. oocytes showed any thymidine incorporation.

At the same time the smaller oocyte packets harvested after two hours exposure showed moderate to heavy labeling over the cytoplasm, confirming what had been found with *in vivo* labeling. None of the packets exhibited any significant nuclear label, which verified the earlier observations that nuclear labeling was confined to cells within the ovary.

In order to substantiate the nature of the thymidine uptake in the cytoplasm, sections of oocytes which had been pulsed for three hours were exposed to DNase

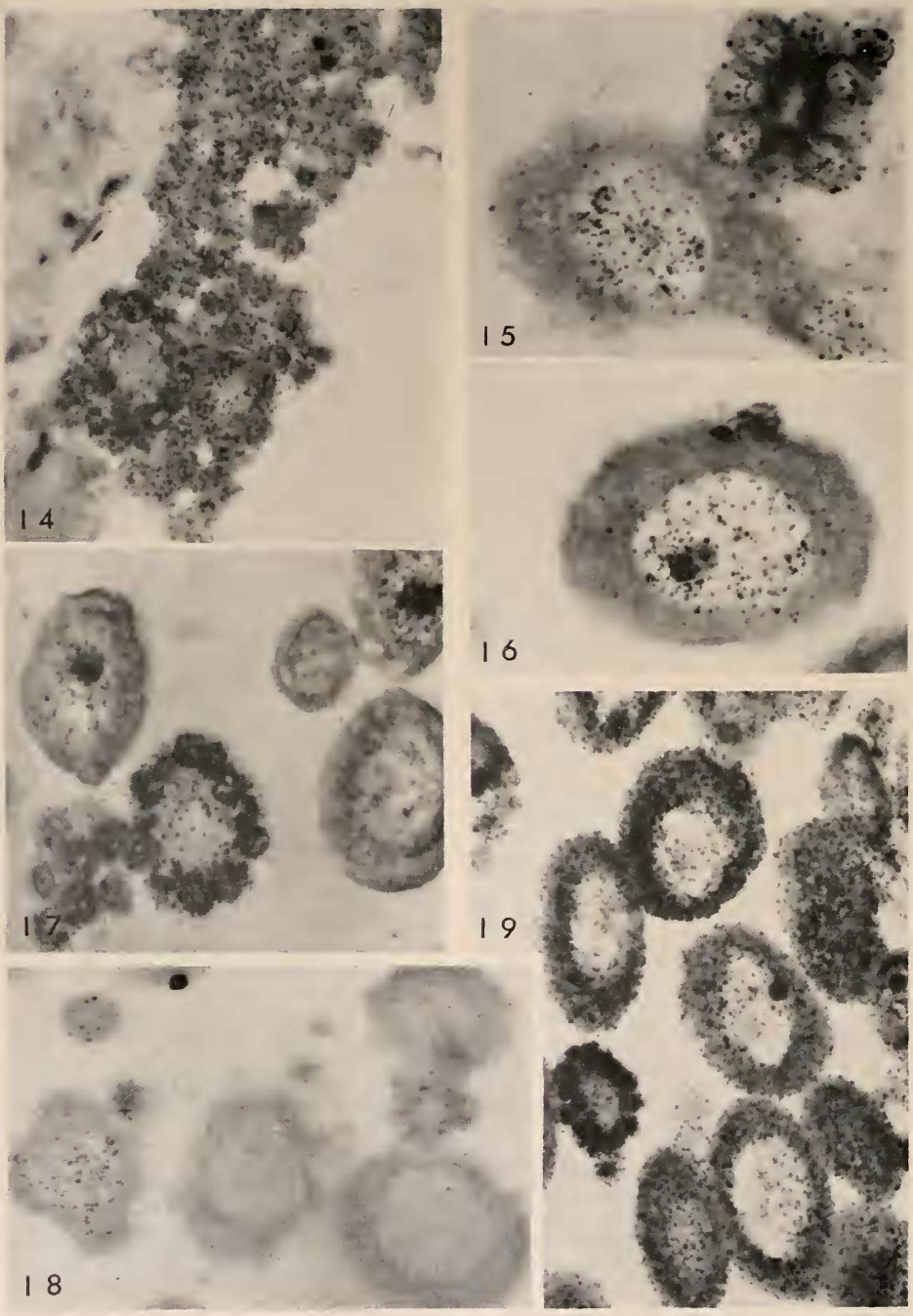


PLATE III

prior to dipping in the emulsion. The DNase treatment completely eliminated any labeling in the cytoplasm of the young oocyte packets.

H^3-uridine

In vivo labeling. Animals were pulsed with either 5 or 10 μC. of H^3-uridine, then the eggs were shed and sectioned or sectioned *in situ*.

The ovary. Unlike the uptake of thymidine in the ovary, H^3-uridine incorporation was evenly distributed throughout the ovary. As soon as 4 hours after injection, the ovary was uniformly labeled over the entire area. Uridine appeared to be taken up equally in the nuclei and cytoplasm of the ovarian cells. The intensity of the label increased with the exposure time so that after 24 hours the ovary exhibited a dense, diffuse distribution of silver grains. Ovaries from animals sacrificed after 48 to 72 hours exposure presented a heavy nuclear label as well as a moderate concentration of grains over the cytoplasm of ovarian oocytes (Fig. 14). After prolonged exposures of 6 days the ovary was still heavily labeled, particularly over the cytoplasm of the oocytes nearest the periphery, while the nuclear concentration had diminished.

Coelomic oocytes. All stages of coelomic oocytes exhibited moderate to heavy nuclear uptake of radioactive uridine after short pulses. While the initial labeling was nuclear, the distribution of the precursor changed with increased exposure.

Oocyte packets. Oocytes exposed to H^3-uridine in the early phase of packet development possessed a moderate to heavy concentration of grains over their nuclei after two hours (Fig. 15). Medium-sized packets were also labeled primarily over the nucleus although a light scattering of grains also appeared over the peripheral or outer cytoplasm. The basal ends which stain heavily for basophilia of the oocytes directed toward the packet center demonstrated little uptake of uridine.

After a 4-hour exposure, the nuclear label became greatly intensified and the cytoplasm accrued additional grain accumulation. Small compact oocyte packets with prominent nuclear labeling also showed diffuse scattered grains, particularly around the peripheral cytoplasmic areas. This same condition was found in oocyte packets after 8 and 24 hours exposure to the uridine. Significantly, in oocyte packets of larger sizes, the granular label was found over the nuclei with scarcely a trace in the cytoplasm.

It was very apparent that the cytoplasmic label first detected in the smaller oocyte groups was intensely distributed over all cell packets, except the large ter-

PLATE III

FIGURE 14. Portion of the ovary with diffuse labeling of oocytes 72 hours after H^3-uridine injection. An oocyte packet is superimposed. 750 ×.

FIGURE 15. Oocyte packet with strong nuclear and light cytoplasmic label adjacent to an individual oocyte. Shed two hours after H^3-uridine injection. 960 ×.

FIGURE 16. Single oocyte with labeled nucleus and heavy concentration of grains over nucleolar bud. Two-hour H^3-uridine pulse. 960 ×.

FIGURE 17. A comparison of the heavy cytoplasmic label in oocyte packets with the predominately nuclear and nucleolar uptake in single oocytes. Sacrified after 72 hours H^3-uridine injection. 936 ×.

FIGURE 18. Coelomic oocytes after two-hour pulse of H^3-uridine and subsequent treatment with RNase. Most cells are unlabeled but occasional nuclei resist treatment and show retention of label. 936 ×.

minal oocyte packets, after 48 hours exposure to H^3-uridine. Presumably, the persistent appearance of the cytoplasmic label in these oocytes either represented utilization of H^3-uridine by RNA or its precursors already in the cytoplasm, or resulted from the movement of RNA from the nucleus into the cytoplasm.

Oocytes in early packet stages continued to display heavy cytoplasmic and nuclear label when the animals were sacrificed 72 hours after injection. Both small and intermediate oocyte packets were affected alike, as seen in Figure 17. Again, in the transition to larger cell packets the grains were still limited to the nuclei with little cytoplasmic concentration.

Single oocytes. After a short pulse (15 to 30 minutes) single cells also gave evidence of H^3-uridine uptake. Two hours after being injected and shed, the smaller single oocytes appeared with a heavy nuclear label (Fig. 15) while larger oocytes showed a less dense nuclear labeling as seen in Figure 16. In some cells sacrificed after 4 hours the nuclear and nucleolar label was so intense that the cells appeared almost black in these areas. There was often a concentration of grains around the nucleoli, particularly above the epinuclear buds (Fig. 16). The cytoplasmic label of scattered single oocytes varied from light to moderate.

Single oocytes which had been shed and recovered 8 hours after injection of the adult animal gave the same pattern. Medium to large single oocytes were heavily tagged over the nucleus, nucleolus and somewhat over the cytoplasm. In the less intensely tagged cells, the nuclei were principally labeled.

Following 24 hours exposure to H^3-uridine, preparations of sectioned oocytes *in situ* indicated that all sizes of single oocytes had a strong nuclear incorporation with scattered grains over the cytoplasm. A survey of 50 fields showed that 81% of these cells possessed a nuclear label.

Sections from H^3-uridine injected animals sacrificed after 48 hours exposure were quite similar and consistent with those recovered after one day.

The same pattern extended to single growing oocytes after 72 hours exposure to uridine. The nuclear labeling of the smallest single oocytes was identical to that seen in the larger oocyte packets. The concentration appeared more intense, particularly around the nucleolus, as the oocytes grew larger and reached a peak in the mature oocytes, while the cytoplasmic uptake was relatively light. This distribution is just opposite to that seen in the young developing oocyte packets which are compared in Figure 17.

Enzyme treatment. Pretreatment of the egg sections with RNase eliminated both the cytoplasmic and nucleolar label after a two-hour pulse with H^3-uridine. While the nuclear label was reduced, scattered single oocytes and packets still possessed a moderately heavy label (Fig. 18). Thus it appeared that a large part of the nuclear label was not susceptible to RNase digestion.

Essentially similar results were obtained in eggs harvested after 8 hours. Most cells, in both packets and single oocyte stages, continued to show a moderate concentration of nuclear grains. Scattered single oocytes showed a heavier label over the nucleus and nucleolus that extended into the cytoplasm as well. Sections of the same eggs were pretreated with RNase before dipping into emulsion. Again this resulted in a complete loss of cytoplasmic and nucleolar labeling as well as the nuclear label from many of the single oocytes. In some of the large single oocytes, however, a heavy nuclear label still persisted after RNase treatment.

Consequently, similar sections were pretreated with DNase but this action did not remove any of the nuclear, nucleolar or cytoplasmic labeling.

Oocytes were harvested and animals were sacrificed at an extended interval of 6 days after injection of tritiated uridine. By the sixth day the intensity of the label had increased considerably. As indicated previously the ovarian oocytes were evenly and densely covered by fine grains. In the coelom, heavy labeling was again seen over the cytoplasm and nuclei of the smaller single oocytes. In larger oocytes the label appeared more concentrated in the karyoplasm of the nucleus and the nuclear membrane, and closely adherent to the nucleolar perimeter. This concentration became more accentuated in the oocytes approaching maturation size. In addition, the cytoplasm of the mature oocytes was evenly and densely covered by fine grains as shown in Figure 19. The outstanding difference noted for single oocytes in all stages of growth was an increase in grains over the cytoplasm. The amount of uptake became so intense that it equalled the original nuclear concentration.

Discussion and Conclusions

The gametes of *Pectinaria* generally were retained in the coelomic cavity over the long period of growth and maturation; only during the latter part of August were gametes found within the lumen of the nephromixia. This suggested that either the ciliated funnels of the nephromixia rejected the gametes until the spawning period or that some mechanism triggered their entrance into the nephromixia. Howie (1961a) observed that changes in the male coelomic gametes of *Arenicola marina* allowed them to be taken up by the nephromixia and subsequently shed. Further experiments with females (Howie, 1961b) indicated that the gonoducts would accept only mature oocytes and that non-spawning individuals could be induced to shed with injections from spawning females.

The proposal is made, based upon the evidence presented here and the observations in *Arenicola* by Howie, that the mature gametes in *Pectinaria* are selected or allowed to enter the nephromixia just prior to spawning. Full maturation of these oocytes, accomplished while the oocytes are still within the nephromixia, is accompanied by changes in the nucleus leading to G.V. breakdown and arrestment in metaphase I (Fig. 5). It is important to note that upon artificial stimulation or release of the oocytes from the coelomic cavity, the same stage in meiosis is reached. More than likely, the low fertility of these artificially shed eggs (Austin, 1963; Tweedell, 1962) is due to premature stimulation (*i.e.,* G.V. breakdown) of these eggs prior to their complete maturation.

During the growth or vegetative period of oocyte development in *Pectinaria* there is a lack of thymidine incorporation into the germinal vesicle, a condition that extends at least to the metaphase of the first maturation division. This response is the same as that in oocytes of the sea urchin (Ficq *et al.,* 1963b), the starfish (Geuskens, 1963), the sand dollar (Simmel and Karnovsky, 1961) and the cricket (Favard-Séréno and Durand, 1963b).

Nuclear uptake of tritiated thymidine and DNA synthesis is apparently restricted to the early generative stage prior to the growth of the germinal vesicle which occurs in the ovary of *Pectinaria.*

The cytoplasm of the early oocyte packets of *Pectinaria* does appear to utilize H^3-thymidine, presumably in the synthesis of cytoplasmic DNA. Furthermore, the labeled material is probably retained in the cytoplasm of the developing oocyte to maturity. This may account for the extra DNA content that has been reported for many eggs (Grant, 1965). In *Ilyanassa,* Collier and McCann-Collier (1962) estimated the amount of DNA of the ovarian eggs to be 32 times that predicted for chromosomal DNA.

Generally, attempts to demonstrate cytoplasmic DNA in frog oocytes by autoradiography have been unsuccessful although Ficq (1961a) did find that the cytoplasm of non-dividing follicle cells became labeled but not the oocyte cytoplasm. In the cricket oocyte, however, the uptake of thymidine by follicular cell cytoplasm was followed by the transfer of radioactive DNA into the ooplasm (Favard-Séréno and Durand, 1963b).

The heavy concentration of radioactive label over the cytoplasm of mature oocytes after a prolonged exposure to H^3-thymidine might be attributed to the accumulation of breakdown products of the original nucleoside. However, the differential uptake of the radioactive thymidine by the cells renders this unlikely. Alternatively, the buildup of cytoplasmic label could result from a migration of previously labeled nuclear DNA after the packets had reached maturity. Just as possible, the collection of cytoplasmic granules could result from cytoplasmic uptake as seen in the early oocyte packet phase with the subsequent fragmentation and development into single mature coelomic oocytes. This seems likely since the cytoplasmic label in the single oocytes appeared only after progressively longer exposures to the H^3-thymidine, finally occurring over 17% of the largest oocytes after 21 days.

The rapid uptake of H^3-uridine into the nuclear sap and particularly the nucleolus of the growing oocytes of *Pectinaria* and the ultimate transfer of the label to the cytoplasm of older oocytes strongly indicated that much of the uridine was used in the production of nuclear RNA. It also supports the contention that most of the cellular RNA used for cytoplasmic growth originates in the nucleus (Prescott, 1960).

Zalokar (1959, 1960) found that after one- to 4-minute exposures of *Neurospora* hyphae to H^3-uridine, followed by centrifugation, only the RNA in the stratified nuclei became labeled. He indicated that at least 99% of the cellular RNA originated in the nucleus. After increased exposures, almost all of the label was found in the cytoplasm.

Ficq (1961a) also found, in autoradiographic studies, that all RNA synthesis in amphibian oocytes is nuclear.

During the diplotene phase of the amphibian oocyte, RNA is actively synthesized along the chromosome. In an autoradiographic study of the newt oocyte, Gall and Callan (1962) found a progressive incorporation of H^3-uridine which moved sequentially along the loops of the lampbrush chromosomes. Microelectrophoretic measurements of the base composition for chromosomal RNA of *Triturus* oocytes indicated low guanine and cytosine, similar to DNA, and suggested m-RNA (Edstrom and Gall, 1963).

Quite similar results to the present study were found in an investigation of free oocytes from the sea urchin *Lytechinus pictus* by Piatigorsky, Ozaki and Tyler

(1966). There was an appreciable amount of C^{14} incorporation in RNA of the oocytes after 15- to 60-minute exposures, measured both quantitatively and by autoradiography. Labeling was found in the germinal vesicle and quite heavily in the nucleolus where uptake was almost entirely blocked by actinomycin D. In mature eggs very little, if any, incorporation of C^{14}-uridine could be measured and none was detected by labeling.

It is likely that much of the labeled nuclear RNA in *Pectinaria* oocytes is m-RNA. Edstrom *et al.* (1961) have shown that the base composition of nuclear RNA in the starfish oocyte resembles DNA and is probably m-RNA. Likewise, Ficq (1961a, 1961b, 1964; Ficq *et al.*, 1963) has long proposed that the uptake of H^3-uridine and H^3-cytidine in the nucleoplasm of various oocytes is due to a high molecular weight, metabolically active RNA, in close contact with DNA, that could be messenger RNA.

More direct evidence of messenger, *i.e.*, chromosomal, RNA was obtained by Sirlin, Jacob and Kato (1962). When a specific block, such as thioacetamide, is applied to the nuclei of chironomid larvae, the nucleolar RNA is inhibited from uptake of H^3-uridine and free m-RNA appears instead. This labeled m-RNA was observed directly in the nucleus and followed to the cytoplasm.

Further indication that H^3-uridine was incorporated into nuclear and nucleolar RNA in the present study resulted when the nucleolar label in all oocytes and part of the nuclear label in most oocytes was removed when they were submitted to RNase prior to autoradiography. Similar autoradiographs of H^3-uridine uptake into the nuclear sap and nucleoli of oocytes have been shown in the cricket (Favard-Séréno and Durand, 1963a), the sea urchin (Ficq *et al.*, 1963), the starfish Geuskens, 1963) and the toad (Ficq, 1964).

The failure of RNase to remove the entire nuclear label from *some* of the larger, single oocytes of *Pectinaria* may be significant since H^3-uridine may act as a precursor for DNA. During vitellogenesis in the cricket oocyte, uridine is incorporated into DNA in the same manner as the uptake of thymidine (Favard-Séréno and Durand, 1963b). Bieliavsky and Tencer (1960) noted that uridine is selectively taken into DNA of amphibian embryos at gastrulation. Collier (1963a, 1965) also found C^{14}-uridine acts as a major precursor in the synthesis of DNA in the *Ilyanassa* embryo.

The same retention of nuclear label was found in H^3-uridine-labeled oocytes of *Pectinaria* after pre-treatment with DNase. If some of the H^3-uridine is converted to DNA, the marked compound must be tied up, possibly as a RNA-DNA hybrid. This awaits further investigation.

The tremendous increase in size, and particularly the budding activity of the nucleoli, during oocyte development and growth in *Pectinaria* also suggest an active production of RNA. Previous results show that both the epinucleolar and intranucleolar buds fluoresce bright red with acridine orange (Tweedell, 1962), another indication of RNA. Nucleolar extrusion and emission of nucleolar products into the cytoplasm is well documented for other oocytes (Raven, 1961). Consequently, the heavy labeling of the nucleolar buds in the primary oocyte of *Pectinaria* supported this viewpoint.

The evidence for nucleolar incorporation of RNA precursors into developing oocytes has been well documented (Ficq, 1961, 1962, 1964; Ficq *et al.*, 1963;

Favard-Séréno and Durand, 1963a; Ozban *et al.,* 1964). Furthermore, Brown and Gurdon (1964) found that there is a total absence of ribosomal RNA synthesis in embryos of an anucleolate mutant of *Xenopus laevis,* even though other RNA was being synthesized.

Yet the relationship between the exact site of nucleolar RNA synthesis, the chromosome and the organizer DNA has not been too clear.

Zalokar (1962) believes the nucleoli are only temporary storage places for newly formed RNA. Following incorporation of H^3-uridine into RNA of oocyte nuclei of *Blatella germanica,* he found that the RNA was detected in the chromosomal region peripheral to the nucleolus. The nucleoli remained unlabeled but chromosomes were labeled when moderate amounts of actinomycin were applied.

In experiments on the salivary gland nuclei of chironomid larvae (*Smittia* sp.) Sirlin *et al.* (1962) pulse-labeled the cells with H^3-uridine and pretreated with TRB, a general RNA inhibitor. The nucleoli were heavily labeled while the nucleolar organizer remained unlabeled. They presented evidence for an extrinsic nucleolar RNA (chromosomal RNA) and an intrinsic nucleolar RNA, and proposed that the chromosomal RNA, not organizer DNA, primed the intrinsic nucleolar RNA.

On the other hand, when isolated amphibian oocytes were exposed *in vitro* to H^3-uridine, H^3-cytidine, incorporation into the lampbrush chromosomes took place (Izawa *et al.,* 1963). However, prior application of actinomycin D blocked uptake in both the chromosomal loops and the nucleolus. From this they concluded that RNA synthesis in both areas is DNA-dependent.

The type of RNA synthesized and the sequential movement of RNA within the nucleus are still very controversial.

One model for the origin of RNA synthesis (Perry, 1965) proposes that the nucleolus accounts for roughly two-thirds of the cytoplasmic RNA (r-RNA species) while extranucleolar synthesis accounts for m-RNA and t-RNA.

It is germane to examine which kinds of RNA are involved in the labeling of the oocyte nucleus and particularly the nucleolus. All three types of RNA (t-RNA, r-RNA and m-RNA) have been recognized in the nucleoli of oocytes and other cells (Sirlin *et al.,* 1963). Direct evidence for two RNA types in the nucleolus was obtained from fractionation of the nucleoli of pea seedlings (Birnstiel and Chipchase, 1963), where it was found that 56% of the extractable RNA is t-RNA (4s), the remainder consisting of ribosomal species.

At least two types of nuclear RNA, which varied in their solubility and specific activity, have been found in the starfish oocyte nucleus and nucleolus (Vincent, 1954, 1957). Vincent and Baltus (1960) later found that the oocyte nucleolus of the starfish binds C^{14}-leucine to an "activation" RNA that is attached to a 4.5s protein, presumably used in the synthesis of cellular proteins.

Possible supporting evidence of nucleolar production of t-RNA was presented by Fleissner and Borek (1962) in the transmethylation of (C^{14} methyl) methionine to ribonucleic acid. Sirlin, Jacob and Tandler (1963) were able to show by autoradiography preferential uptake of (methyl C^{14}) methionine into the nucleolus of the salivary glands of a chironomid. When puromycin was applied to block the incorporation of methionine into the protein of the chromosomes and cytoplasm, the latter showed little or no uptake. The remaining nucleolar label was entirely

removed by RNase. This was interpreted as the transfer of the methyl group to t-RNA of the nucleolus.

The same formation of nucleolar RNA from (methyl C^{14}) methionine has been obtained in developing oocytes of the toad (Ozban, Tandler and Sirlin, 1964). The increase of incorporation into RNase-sensitive nucleolar material was proportional to oocyte growth, *e.g.*, cytoplasmic growth. In older larger oocytes, almost all nucleolar label derived in the presence of puromycin was RNase-sensitive.

In contrast, Ficq (1961) found t-RNA was formed in the cytoplasm of amphibian oocytes from labeled cytidine. More recently, Ficq (1966) combined methyl-C^{14}-methionine with enzymatic digestion by ribonuclease on the oocytes of newts and salamanders. The methylating activity was reported in the cytoplasm as well as the nucleolus; she did not find the localization of t-RNA to be preferentially nucleolar.

Birnstiel, Fleissner and Borek (1963) have reported that RNA methylases are concentrated in the nucleoli of pea nuclei where the enzymes are believed to alter t-RNA by the incorporation of methyl groups into the component bases.

Davidson, Allfrey and Mirsky (1964) concluded that during the lampbrush stage of oogenesis the oocytes of *Xenopus laevis* produce huge quantities of ribosomal RNA. After injection of H^3-uridine, they found that over 90% of the RNA recovered was of the ribosomal type. In the amphibian most of this RNA is produced in early oocytes rather than in ovulated eggs. After isolation of RNA from immature (ovarian) oocytes of *Xenopus* with P^{32} the immature oocytes produced an abundance of two ribosomal species whereas no labeled ribosomal RNA was found in ovulated eggs (Brown and Littna, 1964).

The nucleolar and cytoplasmic RNA in the starfish oocyte have very similar base ratios (Edstrom *et al.*, 1961), suggesting that ribosomal RNA is a result of this nucleolar activity. In oocytes of *Triturus,* Edstrom and Gall (1963) similarly reported there is an overall resemblance between nucleolar and cytoplasmic RNA. Both are rich in guanine-cytosine compared to relatively low G-C content of chromosomal RNA. Furthermore, Ficq (1964) finds that H^3-cytidine, H^3-5-methylcytocine and H^3-uridine are preferentially incorporated into RNA of the oocyte nucleolus of the sea urchin and toad; this uptake is completely blocked by actinomycin D.

Radiography and extraction of RNA from the sea urchin oocytes (Gross *et al.*, 1965) confirm the contention that H^3-uridine is incorporated into several types of RNA during oocyte development. One week after injection of H^3-uridine into *Arbacia punctulata* the whole ovarian region became radioactive. The smaller ovarian oocytes were heavily labeled in both nuclei and cytoplasm while the larger oocytes had radioactivity primarily in the cytoplasm. The number of ootids with a cytoplasmic label varied from $\frac{3}{4}$ of the cells intermediate in the acinus of the ovary to only a few mature ootids from the central lumen. A few were heavily labeled in the cytoplasm and nucleus. Some of the central ootids contained labeled nuclei although "rarely with significant radioactivity." Apparently these were labeled during late maturation stages.

Subsequent extraction of RNA from these central ootids yielded three major RNA components. Two were 28S and 18S ribosomal species; the third was 4S

material, some of which was t-RNA. Another fraction was presumed to be m-RNA.

The relatively light cytoplasmic uptake of H^3-uridine into oocytes of *Pectinaria* after short pulses or moderate exposures indicated that little uridine was taken directly into cytoplasmic RNA. There was, however, a slow increase in cytoplasmic label after two days that increased dramatically after a six-day exposure, which might represent a latent uptake of uridine into ribosomal RNA. However, if nucleolar RNA and ribosomal RNA are related, the cytoplasmic buildup could be due to a migration of nuclear RNA into the cytoplasm. Indeed, the nuclear and nucleolar label does diminish as the cytoplasmic activity increases, a situation similar to that in the cricket oocytes (Favard-Séréno and Durand, 1963a) and in the starfish oocytes (Edstrom *et al.*, 1961).

We can conclude, then, that H^3-uridine is incorporated into several types of RNA, both nuclear and nucleolar in origin, during oocyte development. Presumably the nuclear label is due to m-RNA and ribosomal RNA does appear to be derived directly from nucleolar RNA.

Summary

1. The development and growth of the primary oocytes in the coelomic fluid are described. Oocytes progress from a packet stage to a single oocyte stage accompanied by vegetative growth of the germinal vesicle. Evidence presented indicates that the germinal vesicle breaks down and the oocyte reaches the first maturation division prior to shedding under natural conditions.

2. Nuclear uptake of H^3-thymidine is confined to the ovarian cells following the last oogonial division in the premeiotic phase. There is evidence that free coelomic oocyte packets incorporate H^3-thymidine directly into the cytoplasm. The cytoplasmic label is removed by treatment with DNase.

3. Short pulses of H^3-uridine are taken up diffusely by the ovarian oocytes, while small packets and single oocytes incorporate H^3-uridine primarily in the nucleus and in the nucleolus. Extended exposure from 4 to 48 hours indicates that some of the nuclear uridine moves into the cytoplasm in the packet oocytes. Individual oocytes show strong nuclear labeling up to 72 hours after injection. Particularly long exposures of 6 days indicate that the uridine accumulates in the cytoplasm in the largest oocytes.

4. Treatment with RNase removes most of the nuclear and cytoplasmic H^3-uridine label. Some of the nuclear label is resistant and is not removed by RNase or DNase.

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